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# Antimicrobial and Cytotoxic Activities of Cyanobacteria.

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**Abstract:** Present study screened ten Cyanobacterial extracts for antimicrobial activity, cytotoxicity (against human cervical carcinoma cells Hela and SiHa) & GC-MS for chemical composition. Cyanobacterial extracts were subjected to Agar Well Diffusion Assay at concentration of 100µg. well<sup>-1</sup> and incubated at 37 ± 2°C. Inhibition zone was measured in millimetres (mm) after 18-24 hour. Minimum inhibitory concentration (MIC) was determined by using the broth microdilution method in 96-well microtitre plates. Drug dilutions were performed using cation adjusted Mueller Hinton Broth (MHB) in a concentration range of 128-0.25 µg.ml<sup>-1</sup>. Cytotoxicities were assessed using MTT assay. GC-MS analysis was carried out on a GCMS-QP 2010 Plus Shimadzu system having RTX-5 column (Restek, USA) (60 m, ID 0.25mm, film thickness 0.25 µm). Cyanobacterial extracts exhibited significant antibacterial effect on clinical isolates of *S.aureus* MRSA & MRSE whereas selectively inhibited Gram-negative. Minimum inhibitory concentrations (MIC) were in the range between 64 to 128 µg/ml. Activity Index of active extracts ranged from 0.33 to 1.50. Activity Index and Zone of Inhibition were significantly correlated with p>0.03. GC-MS detected distinct groups of active compounds with pronounced presence of saturated and unsaturated fatty acids. Pharmaceutically important compounds like sesquiterpenoids (Farnesol), dicarboxylic acids, imidazole, indolinones, α-tocopherol, phenolics, phytosterols, heptadecane, tetradecane and 9-octadecenal were moderately present. Extracts also exhibited cytotoxic effect on human cervical carcinoma cells lines with LD<sub>50</sub> values ranging from 34 to 146µg/ml. Cyanobacterial species have distinct active group metabolites which are promising sources of antiproliferative and antimicrobial compounds.

**Keywords:** Cyanobacteria; GC-MS; Antibacterial; cytotoxicity.

## I. INTRODUCTION

Cyanobacteria are morphologically diverse group of Gram-negative eubacteria leading in the development of natural biopharmaceuticals due to presence of structurally diverse groups of compound [1a,b]. Antimicrobial property of natural products have gained importance in recent years due to growing antibiotic resistance and further increase in the infection by antibiotic resistant microorganisms [2a,b]. Present study found chemically diverse groups of compounds among 10 fresh water Cyanobacteria sp. Thin layer chromatographic study showed qualitative relationship as pair affinity (PA) value 50% between *H. fontinalis* and *L. officinalis*, 49.2% between *A. ambigua* and *G. gelatinosa*, 37.5%

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between *Chroococcus* sp., *A. fertilissima*, and *Anabaena* sp., 33.3% between *A. variabilis*, *W. prolifica*, and *Scytonema* sp., 28.6% between *G. gelatinosa*, *A. variabilis*, *W. prolifica*, and *Scytonema* sp. and 16.7% between *A. variabilis*, *W. prolifica*, and *Scytonema* sp. (Fig. 1).

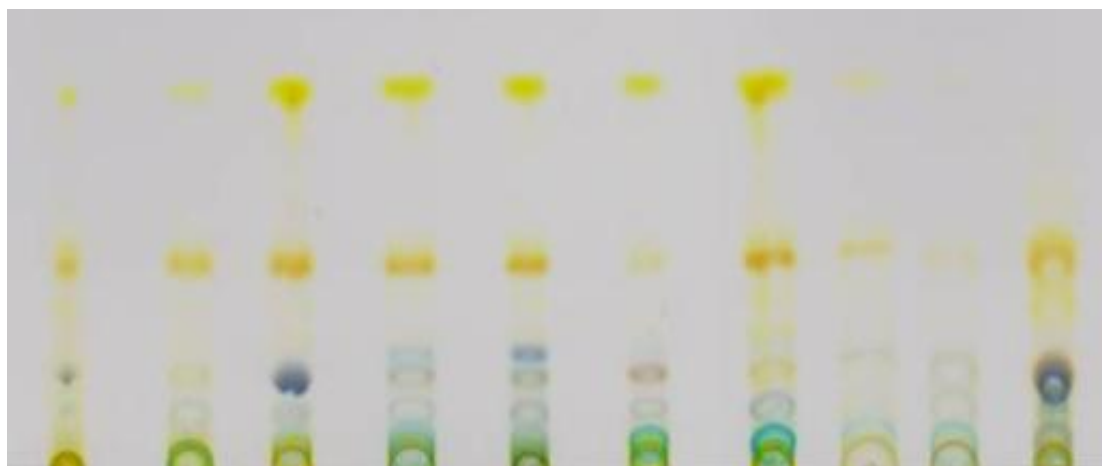


Fig.1 Thin Layer Chromatography of Fresh Water Cyanobacterial Extracts. Lane 1- *H.fontinalis*, 2- *A.ambigua*, 3- *L.officinalis*, 4- *G.gelatinosa*, 5- *Chroococcus* sp., 6- *A.fertilissima*, 7- *Anabaena* sp., 8- *A.variabilis*, 9- *W. prolifica*, 10- *Scytonema* sp.

Highest PA values predict the existence of some common compounds within these species [3]. These relationships were also correlated with the grouped affinity and isolation values of these species. The GC-MS total ion chromatogram of these extracts resolved into several peaks indicating an equally corresponding compounds; 23 in *H. fontinalis*, 21 in *A. ambigua*, 51 in *L. officinalis*, 25 in *G. gelatinosa*, 18 in *Chroococcus* Sp., 34 in *A. fertilissima*, 27 in *Anabaena* Sp., 19 in *A. variabilis*, 19 in *W. prolifica* & 25 in *Scytonema* Sp. (Data not shown here). Principal and pharmacologically active components identified in these extracts included several fatty acids (hydroxy, saturated, branched, unsaturated), tertiary butyl group containing compounds, phenols, terpenoids, phytols, farnesol, hexahydro farnesol, dicarboxylic acids, lactones, heterocyclic compounds, several polyhydroxysterols, nucleoside & complex sugars and natural antioxidant alpha-tocopherol. These components may act synergistically and induce antimicrobial as well as cytotoxic activities. Among fatty acids hexadecanoic acids ( $C_{17}H_{34}O_2$ :  $M_w$ -270), 9,12-octadecadienoic acid ( $C_{19}H_{34}O_2$ :  $M_w$ -294), 9-Octadecenoic acids ( $C_{19}H_{36}O_2$ :  $M_w$ -296), 10 octadecenoic ( $C_{19}H_{36}O_2$ :  $M_w$ -296), eicosanoic ( $C_{21}H_{42}O_2$ :  $M_w$ -326), triacontanoic ( $C_{31}H_{62}O_2$ :  $M_w$ -466) and tetracosanoic acid ( $C_{25}H_{50}O_2$ :  $M_w$ -382) were abundantly present. Benzenedicarboxylic acids such as 1,2-benzenedicarboxylic acid –dioctyl ester ( $C_{24}H_{38}O_4$ :  $M_w$ -390), disooctyl ester ( $C_{24}H_{38}O_4$ :  $M_w$ -390), ethyl hexyl ester ( $C_{24}H_{38}O_4$ :  $M_w$ -278) were also present which are potent antibacterial, antifungal and antialgal agents [4a,b,c]; phytols like 3,7,11(tri); 3,7,11,15(tetra); & 2,6,10; 2,6,11-Trimethyldodecane ( $C_{15}H_{32}$ :  $M_w$ -212) were detected which are known as hexahydrofarnesol and farnesol reportedly possess strong antibacterial activities [5] Imidazole containing alkaloids like 2,4-Imidazolidinedione ( $C_8H_6N_4O_5$ :  $M_w$ -238), 4 amino-5-imidazole carboxymide ( $C_4H_6N_4O$ :  $M_w$ -126) & 1,3-dihydroindol-2-indolinone ( $C_8H_7NO$ :  $M_w$ -133) were present which are reported to possess antimicrobial, cytotoxic, antitumor and antiviral activities [6a,b]. Other

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pharmacologically active compounds detected like 9,12, octadecadienoic acid(C<sub>19</sub>H<sub>34</sub>O<sub>2</sub>:M<sub>w</sub>-294), α-tocopherol(C<sub>29</sub>H<sub>50</sub>O<sub>2</sub>:M<sub>w</sub>-430), and 2,4, di-, tert-butyl phenol(C<sub>14</sub>H<sub>22</sub>O:M<sub>w</sub>-206) have been reported as anti-oxidant [7]; 9,12,15, octadecatrienoic acid (C<sub>20</sub>H<sub>34</sub>O<sub>2</sub>:M<sub>w</sub>-306) have been reported as anti-inflammatory activity [8]; γ-octyl butyrolactones(C<sub>12</sub>H<sub>22</sub>O<sub>2</sub>: M<sub>w</sub>-198) are known as pro-drugs [9]. Several steroidal compounds like dibromostigmasterol acetate (C<sub>31</sub>H<sub>50</sub>Br<sub>2</sub>O<sub>2</sub> : M<sub>w</sub>-612); Stigmast-5-En-3-ol(C<sub>29</sub>H<sub>50</sub>O : M<sub>w</sub>-414); Stigmasta-5,22-Dien-3.beta-ol (C<sub>31</sub>H<sub>50</sub>O<sub>2</sub> :M<sub>w</sub>-454); cholest 5-en-3-ol (C<sub>28</sub>H<sub>45</sub>ClO<sub>2</sub> : M<sub>w</sub>-448 ); Ergost-5-En-3-ol (C<sub>28</sub>H<sub>48</sub>O : M<sub>w</sub>-400); Stigmast-5-En-3-Yl 9-octadecenoate (C<sub>47</sub>H<sub>82</sub>O<sub>2</sub> : M<sub>w</sub>-678) were predominantly present in these extracts (Table 4). These typical sterols are known to posses antibacterial, antifungal and cytotoxic properties. This study was designed to investigate antibacterial activity of Cyanobacterial extracts against 12 Gram-positive and 12 Gram-negative bacteria strains as listed in table I. They were evaluated for their antibacterial potential by agar-well diffusion assay. MICs values were determined further to check the concentration ranges for significant inhibition. Chromatographic separation of the extracts was done by employing TLC. The study was extended to identify the chemical composition of the extracts by Gas Chromatography coupled with Mass Spectrometer (GC-MS) and their cytotoxicity against cervical cancer cell line Hela and SiHa using MTT assay.

## II. MATERIALS AND METHODS

### A. Collection of cyanobacterial culture

Fresh water cyanobacteria (*Lyngbya officinalis* NCCU-102, *Gleocapsa gelatinosa* NCCU-430, *Chroococcus* sp. NCCU-207, *Aulosira fertilissima* NCCU-444, *Anabaena ambigua* NCCU-160, *Hapalosiphon fontinalis* NCCU-339, *Anabaena* sp. NCCU-09, *Anabaena variabilis* NCCU-441, *Westiellopsis prolifica* NCCU-331, and *Scytonema* sp. NCCU-126) were procured from the National Centre for Culture Collection and Utilization of Blue Green Algae, Indian Agriculture Research Institute, New Delhi, India. Cyanobacteria were grown phototrophically in BG-11 medium [10].

### B. Collection of microorganisms

Bacterial strains (Table I) were obtained generously from the culture collections of Ranbaxy Research Laboratories (RRL), Gurgaon, All India Institute of Medical Sciences (AIIMS), New Delhi and National Facility for Marine Cyanobacteria, Department of Marine Biotechnology, Bharathidasan University, India. The test bacterial strains were inoculated on Tryptic Soy Agar (TSA, Difco) and incubated overnight at 37 °C ± 2 °C.

TABLE 1: List of Bacterial Strains Tested For Antimicrobial Activity of Fresh Water Cyanobacterial Extracts.

Bacterial strains	Eq.No.	Origin
<b>Gram Positive</b>		
<i>S.aureus</i> 25923	ATCC 25923	Clinical isolate
<i>S.aureus</i> 562 (MRSA)	ATCC 562	Clinical isolate
<i>S.aureus</i> 1199B	NS	Clinical isolate
<i>S.aureus</i> 209	NS	Clinical isolate
<i>S.aureus</i> MRSA 429	NS	Clinical isolate
<i>S.aureus</i> 2	NS	Clinical isolate
<i>S.epidermidis</i> 35984(MRSE)	ATCC 35984	Skin lesion
<i>S.epidermidis</i> MTCC 435	ATCC 155	Skin lesion
<i>B.subtilis</i> MTCC 2191	NS	NS
<i>B.subtilis</i> NCIM 2068	ATCC 6633	NS
<i>B.subtilis</i> 2620	ATCC 35946	NS
<i>S.aureus</i> NCIM 5021	ATCC 25923	Clinical isolate

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Gram Negative		
<i>E.coli</i> NCIM 2065	ATCC 8739	NS
<i>E.coli</i> 25922	ATCC 25922	Clinical isolate
<i>Aeromonas hydrophila</i> sp.	ATCC 7966	Fish
<i>Klebsiella pneumonia</i> MTCC3384	ATCC 13883	NS
<i>Proteus vulgaris</i> MTCC426	ATCC 6380	NS
<i>Pseudomonas aeruginosa</i> NCIM 5029	ATCC27853	NS
<i>Pseudomonas cepacia</i> MTCC 7099	BAM-6	Soil
<i>Serratia</i> sp.	NiVa-51	River
<i>Serratia marcescens</i> MTCC 97	ATCC 13880	Pond water
<i>Vibrio harveyi</i> MTCC 3438	ATCC 11318	NS
<i>Vibrio parahaemolyticus</i> MTCC 451	NS	NS
<i>Proteus mirabilis</i> MTCC	NS	NS

\*ATCC: American Type Culture collection; MTCC: Microbial Type Culture Collection; NCIM: National Collection of Industrial Microorganisms; D-days; NS – Not specified.

**C. Cyanobacterial biomass harvesting and extraction**

Cyanobacterial late exponential growth phase culture were harvested by centrifugation at 5000 x g for 10 min. Biomass of Cyanobacteria were lyophilized (Labconco, USA) and crushed to fine powder. Fine powder of biomass and diethyl ether solvent were mixed in 100 ml flask maintained in ice bath and sonicated using small probe (Sonics Vibracell, USA), for 2 min, time interval of 5 sec, at 10% of total amplitude and left for 30 min. Extracts were filtered through Whatman No.1 filter paper and solvent was dried in rotary evaporator (Buchi, USA). Dry residue was transferred to a freeze drying vessel and used for different assays by the clearance of bio-safety and ethical committee of the institute.

**D. Thin layer chromatography**

Chromatography was performed on silica gel 60 F<sub>254</sub> TLC aluminium sheet 20x20 cm (Merck). A 50µl sample (1mg/ml) was spotted manually on silica gel and run in mobile phase, hexane: ethyl acetate (8.5:1.5). Numbers of spots were counted and retention factors (R<sub>F</sub>) of each spot was calculated. Qualitative relationships of the each spots were calculated as values of paired affinity, group affinity and isolation value.

**E. Gas chromatography – Mass spectrometry**

Cyanobacterial extracts were subjected to GC-MS analysis on a GCMS-QP 2010 Plus Shimadzu system (All India Research Facility, JNU, Campus, New Delhi) comprising an auto injector and RTX-5 column (Restek, USA) (60 m, ID 0.25mm, film thickness 0.25 µm). Helium was used as the carrier gas (1ml/min). The column temperature was programmed at 60°C for 1 min and then raised to 180°C for the total analysis time of 50 min. Sample (0.1mL) was injected in the splitless mode. The chemical components of the extracts were identified by comparing the retention time of the chromatographic peaks with that of authentic compound using the WILEY8.LIB and NIST05s.LIB.

**F. Agar well diffusion assay**

Antimicrobial assay of dried Cyanobacterial extracts (reconstituted in 1% aq. DMSO) against the bacteria were assessed using the National Committee for Clinical Laboratory Standards guidelines (NCCLS) for agar well diffusion assay [11]. Inhibition zone was measured in millimetres (mm) after 24 hour. 1% DMSO used as negative control and standard antibiotic, Vancomycin (Eli Lilly), Ciprofloxacin and Kanamycin (HiMedia) were used as positive control. Antimicrobial index was calculated as: Inhibition zone of test sample /Inhibition zone of standard.

**G. Minimum Inhibitory Concentration (MIC) by broth microdilution assay**

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MIC of Cyanobacterial extracts was determined by using the broth microdilution method as recommended by NCCLS [12]. The tests were performed in sterile 96-well microtitre plates. Drug dilutions were performed using cation adjusted Mueller Hinton Broth (MHB, Difco) to get a concentration range of 0.25 -128  $\mu\text{g}.\text{ml}^{-1}$ . Inoculum was prepared by direct colony suspension in normal saline to get an inoculum size  $1-2 \times 10^6 \text{ CFU}.\text{ml}^{-1}$ . 100  $\mu\text{l}$  of the inoculum was added in each wells containing 100 $\mu\text{l}$  extracts dilution (final inoculum was approx.  $3-7 \times 10^5 \text{ cfu/ml}$ ). For positive control extracts free inoculated broth and for negative controls sterile broth were used. The final volume in each well was 200  $\mu\text{l}$ . Plates were incubated for 24 hours at  $37 \pm 2^\circ\text{C}$  and observed for presence or absence of growth. The lowest concentration of the extracts that prevented the growth of bacteria was considered to be the MIC.

## H. Cytotoxicity Assay

Human cervical cancer cell lines SiHa and HeLa were obtained from National Centre for Cell Sciences (NCCS) Pune, India. Cell lines were grown as monolayer cultures in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 mg L<sup>-1</sup> streptomycin) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Cytotoxicity of Cyanobacterial extracts were carried in overnight seeded cells ( $2 \times 10^4$ /well) for 48 h. At the end of the treatment, the medium was removed and cells were incubated with 20 $\mu\text{l}$  of MTT (5 mg/ml in PBS) in fresh medium for 4 hrs at 37°C. After four hours, formazan crystals, formed by mitochondrial reduction of MTT, were solubilized in DMSO (200 $\mu\text{l}$ /well) and the absorbance was read at 570 nm after 10 min incubation on the iMark Microplate Reader (Bio-Rad, USA) [13a,b]. Cytotoxicity of Cyanobacterial species was expressed as LC<sub>50</sub>.

## I. Statistical analysis

The mean value  $\pm$  standard error of mean (SEM) was determined from triplicate samples for each experimental group using using Graph Pad prism 2.01 (2) version for Windows 2001 (Graph Pad Software, San Diego). Statistical significance was examined with Student's *t* test.

## III. RESULTS AND DISCUSSIONS

The antibacterial activities (zone of inhibition; respective activity index and MIC values) of each extracts against Gram-positive and Gram-negative bacteria including MRSA & MRSE are summarized in tables II a,b & III. Cyanobacterial extracts significantly inhibited Gram-positive bacteria, whereas selectively inhibited Gram-negative bacteria. It was noted that the cyanobacterial extracts inhibited all tested *Staphylococcal* isolates. Maximum inhibition zone in millimetre was observed in case of *A. ambigua* (37.73), *A. variabilis* (33.36), *Scytonema* Sp. (30.15) and *A. fertillisma* (30.01) against *S.aureus* 25923. However, highest activity index was in order of *A. variabilis*, *A. fertillisma*, *Anabaena* sp., *A. ambigua*, and *Scytonema* Sp. against MRSA (Methicillin Resistant *Staphylococcus aureus*) 562 with MICs of 64 $\mu\text{g/ml}$ . Hence, this strong activity index of cyanobacterial extract directly correlate its antimicrobial potential. Whereas, towards Gram-negative bacteria highest activity index was shown only by *A. ambigua* and *A. variabilis* against *V. parahemolyticus* 451 and *Serratia* sp., while *L. officinalis* showed highest activity index against *Proteus mirabilis*. & *Pseudomonas cepacia*. Zone of Inhibition and Activity Index of each active extracts were significantly correlated ( $p, \leq 0.03$ ). Minimum inhibitory concentrations (MIC) of some of the most active extracts range between 64 $\mu\text{g/ml}$  to 128 $\mu\text{g/ml}$ , are shown in table 3. It was noted that lower MIC (64 $\mu\text{g/ml}$ ) was obtained only against *S.aureus* 25923 and *S.aureus* 562 treated with *A. ambigua*, *L. officinalis*, *G. gelatinosa*, *A. fertillisma*, *A. variabilis*, *W. prolifica*, and

TABLE IIa : Zones of Inhibition Produced by Extracts in the Agar Well Diffusion Assay. (Mean of Three Experiments)

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Bacterial strains	Cyanobacterial Strains												
Gram Positive	1	2	3	4	5	6	7	8	9	10	11	12	13
<i>S.aureus</i> 25923	14.03 ± 0.15 (0.38)	37.73 ± 0.06 (1.04)	18.07 ± 0.06 (0.5)	16.15 ± 0.05 (0.44)	16.06 ± 0.03 (0.44)	30.04 ± 0.01 (0.84)	20.16 ± 0.56 (0.56)	33.36 ± 0.55 (0.92)	25.93 ± 0.10 (0.72)	30.15 ± 0.05 (0.83)	36±0.02	NT	NT
<i>S.aureus</i> 562	10.13 ± 0.11 (0.28)	20.0 ± 0.05 (1.25)	18.01 ± 0.01 (1.12)	18.11 ± 0.01 (1.13)	T	22.09 ± 0.01 (1.38)	20.25 ± 0.13 (1.26)	24.09 ± 0.16 (1.5)	15.97 ± 0.06 (0.99)	20.0 ± 0.04 (1.25)	16±0.01	NT	NT
<i>S.aureus</i> 1199B	-	15.99 ± 0.01 (0.79)	T	T	-	8.03 ± 0.02 (0.4)	-	16.03 ± 0.01 (0.8)	14.08 ± 0.01 (0.7)	11.98 ± 0.02 (0.59)	20±0.01	NT	NT
<i>S.aureus</i> 209	-	16.03 ± 0.05 (0.76)	16.11 ± 0.09 (0.76)	18.11 ± 0.02 (0.86)	T	22.02 ± 0.01 (1.04)	T	16.03 ± 0.03 (0.76)	19.74 ± 0.65 (0.94)	-	21±0.02	NT	NT
<i>S.aureus</i> 429	-	-	10.07 ± 0.06 (0.5)	-	-	-	-	12.13 ± 0.05 (0.6)	10.1 ± 0.01 (0.5)	14.0 ± 0.02 (0.7)	20±0.02	NT	NT
<i>S.aureus</i> 2	12.24 ± 0.05 (0.38)	14.13 ± 0.11 (0.44)	18.14 ± 0.01 (0.56)	13.88 ± 0.12 (0.43)	18.05 ± 0.05 (0.56)	12.11 ± 0.01 (0.37)	-	T	T	20.05 ± 0.06 (0.62)	32±0.01	NT	NT
<i>S.epidermidis</i> 35984	-	12.03 ± 0.05 (0.4)	13.67 ± 0.5 (0.45)	11.91 ± 0.07 (0.39)	10.5 ± 0.05 (0.35)	-	-	T	T	-	30±0.02	NT	NT
<i>S.epidermidis</i> 435	-	-	13.98 ± 0.02 (0.77)	15.00 ± 0.01 (0.83)	T	9.0 ± 0.10 (0.5)	8.96 ± 0.15 (0.49)	T	12.07 ± 0.06 (0.67)	10.04 ± 0.05 (0.55)	18±0.01	NT	NT
<i>B.subtilis</i> 2191	-	12.00 ± 0.01 (0.6)	-	-	-	9.03 ± 0.05 (0.45)	-	9.00 ± 0.01 (0.45)	-	10.03 ± 0.05 (0.5)	10±0.01	20±0.01	NT
<i>B.subtilis</i> 2068	-	-	-	-	-	10.98 ± 0.01 (0.27)	11.02 ± 0.02 (0.27)	14.92 ± 0.06 (0.37)	15.92 ± 0.06 (0.4)	10.02 ± 0.01 (0.38)	19±0.01	40±0.01	NT
<i>B.subtilis</i> 2620	-	10.99 ± 0.01 (0.6)	-	-	-	9.96 ± 0.05 (0.33)	12.00 ± 0.01 (0.4)	14.99 ± 0.01 (0.49)	13.07 ± 0.06 (0.49)	12.03 ± 0.05 (0.4)	30±0.01	-	NT
<i>S.aureus</i> 5021	-	14.00 ± 0.01 (0.46)	-	-	-	-	-	-	-	-	NT	30±0.01	NT

1-*H. fontinalis*, 2-*A. ambigua*, 3-*L. officinalis*, 4-*G. gelatinosa*, 5-*Chroococcus* sp., 6-*A. fertilissima*, 7-*Anabaena* sp., 8-*A. variabilis*, 9-*W. prolifica*, 10-*Scytonema* sp. Activity index (in parenthesis) is calculated with respect to standard positive control. 6.25µg/ml of Vancomycin (11), Ciprofloxacin (12), and Kanamycin (13) used as standard positive control.

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TABLE IIb : Zones of Inhibition Produced by Extracts in the Agar Well Diffusion Assay.(Mean of Three Experiments)

Bacterial strains	Cyanobacterial Strains												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Gram Negative													
<i>E.coli</i> 25922	-	-	-	-	-	-	-	-	-	-	-	18 ± 0.02	20 ± 0.02
<i>E.coli</i> 2065	-	9.96 ± 0.06 (0.49)	-	-	-	-	-	-	9.01 ± 0.02 (0.45)	7.01 ± 0.01 (0.35)	NT	18 ± 0.01	20 ± 0.01
<i>A. hydrophila</i> sp.	-	6.93 ± 0.05 (0.27)	-	-	-	-	-	-	-	-	NT	NT	25 ± 0.02
<i>K. pneumonia</i> 3384	-	12.00 ± 0.01 (0.54)	-	-	-	-	-	-	-	-	NT	NT	22 ± 0.01
<i>P. vulgaris</i> 426	-	-	-	-	-	-	-	-	-	-	NT	NT	18 ± 0.01
<i>P. aeruginosa</i> 5029	-	-	-	-	-	-	-	-	-	T	NT	NT	-
<i>P. cepacia</i> 7099	-	-	8.00 ± 0.01 (0.53)	-	-	-	-	-	-	-	NT	NT	15 ± 0.02
<i>Serratia</i> sp.	-	10.03 ± 0.05 (1)	-	-	-	-	-	10.03 ± 0.05 (1)	-	-	NT	NT	-
<i>S. marcescens</i> 97	-	-	-	-	-	-	-	±	-	-	NT	NT	10 ± 0.01
<i>V. harveyi</i> 3438	-	-	T	-	-	-	-	±	-	T	10 ± 0.01	NT	-
<i>V parahamolyticus</i> 451	-	12.07 ± 0.06 (1)	-	-	-	-	-	10.08 ± 0.03 (0.84)	-	10.13 ± 0.15 (0.84)	12 ± 0.01	NT	T
<i>P. mirabilis</i>	-	-	9.03 ± 0.05 (0.75)	-	-	-	-	-	-	-	10 ± 0.01	NT	12 ± 0.01

1-*H. fontinalis*, 2-*A. ambigua*, 3-*L. officinalis*, 4-*G. gelatinosa*, 5-*Chroococcus* sp., 6-*A. fertilissima*, 7-*Anabaena* sp., 8-*A. variabilis*, 9-*W. prolifica*, 10-*Scytonema* sp. Activity index (in parenthesis) is calculated with respect to standard. 6.25µg/ml of Vancomycin (11), Ciprofloxacin (12), and Kanamycin (13) used as standard positive control.

TABLE III: Minimum Inhibitory Concentration (MIC) of Cyanobacterial Extracts.

Microorganisms	Cyanobacterial extracts	Antibiotic
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<u>Gram Positive</u>	1	2	3	4	5	6	7	8	9	10	Vanco*
<i>S.aureus</i> 25923	128	64	64	64	128	64	64	64	128	64	2
<i>S.aureus</i> 562 (MRSA)	128	64	64	64	>128	64	64	64	128	64	4
<i>S.aureus</i> 1199B	-	128	>128	>128	-	128	-	128	128	128	2
<i>S.aureus</i> 209	-	128	128	128	>128	64	-	128	128	-	2
<i>S.aureus</i> MRSA 429	-	-	128	-	-	-	-	128	128	128	2
<i>S.aureus</i> 2	128	128	64	128	128	128	-	>128	>128	64	2
<i>S.epidermidis</i> 35984(MRSE)	-	128	64	128	128	-	-	>128	>128	-	2
<i>S.epidermidis</i> MTCC 435	-	-	128	128	>128	128	128	>128	-	128	2
<i>B.subtilis</i> MTCC 2191	-	128	-	-	-	128	-	128	-	128	4
<i>B.subtilis</i> NCIM 2068	-	-	-	-	-	128	128	128	128	128	4
<i>B.subtilis</i> 2620	-	128	-	-	-	128	128	128	128	128	4
<i>S.aureus</i> NCIM 5021	-	128	-	-	-	128	-	-	-	-	2
<u>Gram Negative</u>											Cipro*
<i>E.coli</i> NCIM 2065	-	-	-	-	-	-	-	-	128	128	2
<i>E.coli</i> 25922	-	-	-	-	-	-	-	-	-	-	2
<i>A. hydrophila</i> sp.	-	-	-	-	-	-	-	-	-	-	4
<i>K. pneumonia</i> MTCC3384	-	-	-	-	-	-	-	-	-	-	4
<i>Proteus vulgaris</i> MTCC426	-	-	-	-	-	-	-	-	-	-	2
<i>P. aeruginosa</i> NCIM 5029	-	-	-	-	-	-	-	-	-	>128	2
<i>P. cepacia</i> MTCC 7099	-	-	128	-	-	-	-	-	-	-	2
<i>Serratia</i> sp.	-	128	-	-	-	-	-	128	-	-	2
<i>Serratia marcescens</i> MTCC 97	-	-	-	-	-	-	-	-	-	-	2
<i>Vibrio harveyi</i> MTCC 3438	-	-	-	-	-	-	-	-	-	>128	2
<i>V parahaemolyticus</i> MTCC 451	-	128	-	-	-	-	-	128	-	128	2
<i>Proteus mirabilis</i> MTCC	-	-	128	-	-	-	-	-	-	-	2

\* Vancomycin (vanco) and Ciprofloxacin (Cipro) used as positive control. 1-*H. fontinalis*, 2-*A. ambigua*, 3-*L. officinalis*, 4-*G. gelatinosa*, 5-*Chroococcus* sp., 6-*A. fertilissima*, 7-*Anabaena* sp., 8-*A. variabilis*, 9-*W. prolifica*, 10-*Scytonema* sp.

*Scytonema* sp.; against *S.aureus* 2 and *S. epidermidis* 35984 treated with *Scytonema* sp. and *L. officinalis* respectively. MICs values of bioactive extracts and its zone of inhibition were significantly correlated with p, ≤0.05. The antimicrobial property of cyanobacterial species also significantly correlated with the paired affinity (PA) relation. Cyanobacterial stains extracts also showed cytotoxic against human cervix cancerous cell lines. The cytotoxicity trend showed steep rises with the initially increase of concentration and further became constant at high doses. LD<sub>50</sub> against HeLa and SiHa cell lines (Fig. 2)



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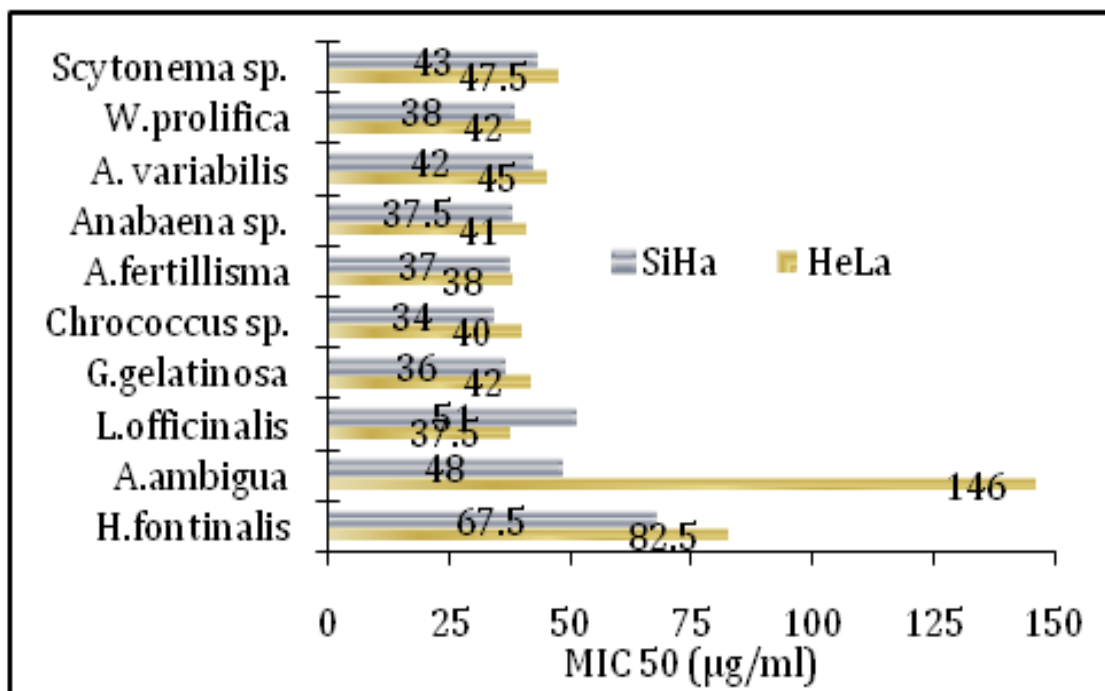


Fig. 2. Cytotoxicity of cyanobacterial extracts against SiHa and HeLa cell lines (Data represented as mean of three experiments).

Comparatively, highest cytotoxicity even at low concentration was observed in *L. officinalis*, *G. gelatinosa*, *Chrococcus* sp., *A. fertillisma*, *Anabaena* sp. and *A. variabilis* extracts, whereas *A. ambigua* treated HeLa cell line showed lowest cytotoxicity (LD<sub>50</sub> 146µg/ml). The GC-MS total ion chromatogram of these extracts resolved into several peaks indicating an equally corresponding compounds; 23 in *H. fontinalis*, 21 in *A. ambigua*, 51 in *L. officinalis*, 25 in *G. gelatinosa*, 18 in *Chrococcus* Sp., 34 in *A. fertillisma*, 27 in *Anabaena* Sp., 19 in *A. variabilis*, 19 in *W. prolifica* & 25 in *Scytonema* Sp.(data not shown here).Several important chemicals were identified by GC-MS (Table IV).

TABLE IV: Active Compounds (in % Relative Content) Present in Cyanobacterial Species.

Active compounds	Cyanobacterial Species									
	1	2	3	4	5	6	7	8	9	10
Hexadecanoic acid		17.								
	20.1	4	2.4	8.0	9.17	9.4	12.1	20.3	18.9	16.5
9,12-octadecadienoic acid	9.6	16.	8.5	7.90	8.75	8.39	10.6	17.9	-	9.47
		3					9	0		
9-Octadecenoic acid	10.4	-	4.1	10.4	10.6	11.0	14.9	27.5	22.9	26.0
	4		3	9	1	3	3	3		2
10-Octadecenoic acid	-	24.	-	-	-	-	-	-	-	-
		6								

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Eicosanoic acid	1.0	2.2	-	-	0.87	-	-	-	-	-
		7								
Triacotanoic acid	1.20	-	-	-	-	-	-	-	-	-
Tetracosanoic acid	1.10	4.1	-	-	-	0.48	-	-	2.6	0.78
		7								
Docosanoic acid	-	0.4	-	-	-	0.62	-	-	-	0.77
		5								
Benzenecarboxylic acid dioctylester	2.81	-	-	1.20	3.67	-	6.1	-	1.9	11.2
										2
Benzenecarboxylic acid disoctylester	-	-	12.	-	-	-	-	-	-	-
			6							
Benzenecarboxylic acid bis 2 -ethyl hexyl	-	-	-	-	0.56	10.6	-	4.55	1.58	-
						4				
3,7,11,15-Tetramethyl-2-hexadecene-1-ol	-	2.0	1.7	0.87	2.92	1.27	3.62	-	-	0.4
			8							
3,7,11-Trimethyldodecane(HexahydroFarneso l)	-	-	-	1.21	-	-	-	-	-	-
2,6,11-Trimethyldodecane(Farnesol)	-	6.3	-	-	-	0.50	-	-	1.1	-
		7								
2,6,10-Trimethyl-14-pentadecene	2.8	4.4	1.8	15.4	-	0.52	-	-	1.07	2.17
		8	3							
2,4-Imidazolidinedione	0.57	-	-	-	-	-	-	-	-	-
4-Amino-5-Imidazolecarboxamide	-	-	0.3	-	-	-	-	-	-	-
			3							
1,3-Dihydroindole-2-indolinone	-	-	1.3	-	-	-	-	-	-	-
			6							
$\alpha/\gamma$ Tocopherol	-	-	4.1	1.87	-	1.2	1.24	-	-	0.98
			5							
2,4-Di-Tert.Butylcyclohexanecarboxylic acid	0.64	-	2.4	8.09	-	-	5.1	-	1.81	-
			5							
$\gamma$ -Octyl-Butyrolactone	1.1	0.6	-	-	-	-	-	-	-	-
		2								
$\gamma$ -Heptylbutyrolactone	0.38	-	-	-	-	-	-	-	-	-
$\gamma$ -Hexylbutyrolactone	2.12	-	-	-	-	-	-	-	-	-
22,23-Dibrobestigmasterol	12.8	-	-	-	-	1.18	-	-	-	-
		6								
Stigmast-5-en-3-ol( $\beta$ -Sitosterol)	9.18	1.2	3.6	21.5	4.6	3.3	8.82	-	5.46	-
				3						
Stigmast-5,22-dien-3-ol ( $\beta$ -Stigmasterol)	1.25	1.5	3.1	3.06	-	5.25	-	0.72	-	-
		4	8							
Cholest-5-en-3-ol	3.9	-	1.1	-	-	-	-	1.02	0.45	4.15
			4							
Ergost-5-en-3-ols (campesterol)	-	1.2	1.1	-	-	-	-	-	-	-
		3	4							

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Stigmast-5-en-3-yl-9-octadecanoate	-	0.5	4.78	4.57	3.27	-	6.34	-	-
		8							
5-Heptyldihydro-3-h-Furanone	-	0.5	-	-	-	-	1.17	-	-
		7							
3,5-Dihydro-methyl-dihydropyran-4-one	-	-	4.4	-	-	-	-	-	-
		3							
5-Hydroxy methyl-2-Furancarboxyaldehyde	-	-	8.9	-	-	2.10	-	-	-
		6							
1,4-Benzenediol- $\alpha$ -hydroquinone	-	-	5.7	-	-	-	-	-	-
		9							
p-Hydroquinone	-	-	-	-	4.30	-	-	-	-
1,6-Anhydrodeoxyglucopyranose	-	-	-	-	3.62	-	-	4.73	-
1,5-Anhydro-deoxyhexodilulose	-	-	-	-	4.27	-	-	3.49	-
2,O-Methyl-D-Mannopyranosa	-	-	-	-	7.56	-	-	-	-
Heptadecane /decanoic ester	-	-	0.3	-	-	-	20.9	-	-
		3					5		
Tetradecane/decanoic ester	-	-	-	-	0.56	-	-	-	1.02
Glycerol esters	-	-	-	-	34.4	5.12	1.21	0.85	-
					3				
Citronellyl esters	1.21	-	-	-	-	-	-	0.61	-
9-Octadecenal	-	-	-	0.98	-	1.17	-	2.90	25.6
									0
Octadecanthiol	-	-	1.9	-	0.56	-	-	-	-
		9							
1,Napthalenepentenoic acid	--	-	2.1	-	-	-	-	-	-
		6							
$\alpha$ -Scopolamine	-	-	0.7	-	-	-	-	-	-
		2							

1-*H. fontinalis*, 2-*A. ambigua*, 3-*L. officinalis*, 4-*G. gelatinosa*, 5-*Chroococcus* sp., 6-*A. fertilissima*, 7-*Anabaena* sp., 8-*A. variabilis*, 9-*W. prolifica*, 10-*Scytonema* sp.

These extracts contained 03 hydroxy acids (1.51 & 0.49% relative content in *L. officinalis* & *Chroococcus* sp); 07 saturated and 10 unsaturated fatty acids (Table V). The percent relative content for both types of fatty acids was as high as 23.36 and 46.47 % in *H. fontinalis* and *A. variabilis* respectively. The abundance of saturated fatty acids like palmitic (20.1, 17.4, 20.3, 18.9 & 16.5%) & myristic acid (0.5 & 1.02%) and the unsaturated fatty acids palmitoleic (9.01%), oleic (4.13 – 27.53 %), linoleic (7.9-17.90%) and  $\alpha$ -linolenic(0.74%) acids present in respective cyanobacterial species correlates their significant antibacterial action. Of these unsaturated fatty acids the antimicrobial activity of  $\alpha$ -linolenic acid is well established. [14] A moderate content of three isomers of benzenedicarboxylic acid (dioctyl, disooctyl and ethylhexyl) were present in each extract except in *A. ambigua*. These are known as phthalate derivatives; they exhibit a pronounced anti-bacterial action and have been reported by various investigators as cited earlier from different sources. Among these compounds, 1, 2-Benzenedicarboxylic acid bis(2 ethyl

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TABLE V: Fatty Acids (% Relative Content) Present in Cyanobacterial Species.

Fatty acids	Cyanobacterial Species									
	1	2	3	4	5	6	7	8	9	10
<b>Hydroxy</b>										
2-Hydroxy-1hydroxy methyl Hexadecanoic acid	-	-	0.45	-	-	-	-	-	-	-
17-Hydroxy 6-pent -3-oic-acid	-	-	1.06	-	-	-	-	-	-	-
1-Hydroxy 1-Methyl-2 Ethanediyl Hexadecanoic	-	-	-	-	0.49	-	-	-	-	-
Total Hydroxy Fatty acids	-	-	1.51	-	0.49	-	-	-	-	-
<b>Saturated</b>										
Tetradecanoic	-	-	-	-	-	0.56	-	-	-	1.02
Hexadecanoic	20.1	17.4	2.4	8.0	9.17	9.4	12.1	20.3	18.9	16.5
Heptadecanoic	-	-	-	-	-	-	-	1.29	-	-
Eicosanoic acid	0.96	2.27	-	-	0.87	-	-	-	-	-
Docosanoic	-	0.45	-	-	-	0.62	-	-	-	0.77
Tetracosanoic	1.10	4.17	-	-	-	0.48	-	-	2.55	0.78
Triacotanoic	1.20	-	-	-	-	2.7	-	-	-	-
Total Saturated Fatty acids	23.36	24.29	2.4	8.0	10.04	13.76	12.1	21.59	21.45	19.07
<b>Unsaturated</b>										
7-Hexadecenioc (Palmitoleic acid)	9.01	-	-	-	-	-	-	-	-	-
9-Hexadecenoic Palmitoleic acid)	-	-	0.76	-	-	-	-	-	-	-
9-Heptadecanoic (Myristoleic acid)	-	0.75	0.33	-	-	-	-	-	-	3.18
1-Octadecenoic(Oleic acid)	-	-	0.50	-	-	-	-	-	-	-
9-Octadecenoic (Oleic acid)	10.44	-	4.13	10.49	10.61	11.03	14.93	27.53	22.9	26.02
10-Octadecenoic(Oleic acid)	-	24.62	-	-	-	-	-	-	-	-
9,12-Octadecadienoic(Linoleic acid)	9.6	16.3	8.5	7.90	8.75	8.39	10.69	17.90	-	9.47
8,11- Octadecadienoic (Linoleic acid)	-	-	-	-	-	-	-	-	-	0.61
9,12,15-Octadecatrienoic ( $\alpha$ -Linolenic)	-	-	-	-	-	0.74	-	-	-	-
15-Octadecenoic acid(linoleic)	-	-	-	-	-	-	-	1.04	-	-
Total Unaturated Fatty acids	29.05	41.67	14.22	18.39	19.36	20.16	25.62	46.47	22.9	39.28

1-*H. fontinalis*, 2-*A. ambigua*, 3-*L. officinalis*, 4-*G. gelatinosa*, 5-*Chroococcus* sp., 6-*A. fertillisma*, 7-*Anabaena* sp., 8-*A. variabilis*, 9-*W. prolifica*, 10-*Scytonema* sp.

hexyl) has potent antibacterial activity and was present in *Chroococcus* Sp. (0.56%), *A. Fertillisma* (10.64%), *A. Variabilis* (4.55%) & *W. prolifica* (1.58%) which also showed the higher zone of inhibitions (16.06, 30.04, 33.36

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& 25.93 mm) and lower MIC of 64 to 128  $\mu\text{g/ml}$  against *S. aureus* 25923. Among the other two isomers of these phthalates, diisooctyl was detected only in *L. officinalis* (12.6%); and dioctyl was detected in *H. fontinalis* (2.81%), *G. gelatinosa* (1.20%), *A. Fertillisma* (3.67%), *Anabaena* Sp. (6.1%), *W. prolifica* (1.9%) and *Scytonema* Sp. (11.2%). The phytols, also known as terpene alcohols, were present as lower contents in *H. fontinalis* (2.8%), *L. officinalis* (3.61%), *Chroococcus* Sp. (2.92%), *A. fertillisma* (2.29%), *Anabaena* Sp. (3.62%), *W. prolifica* (2.08%) and *Scytonema* Sp. (2.57%) while it was present in high amounts in *G. gelatinosa* (17.48%), *A. Ambigua* (12.85%). Terpene alcohols, also known as sesquiterpenes were also present in the extracts. In the extracts of *A. Ambigua*, *A. Fertillisma* & *W. prolifica*; a sesquiterpenoid known as farnesol which is antibacterial in its action was present as 6.37, 0.50 & 1.1% respectively. Besides this, trimethyl alkanes (3,7,11-dodecane) & tetramethyl alkenes (3,7,11,15-hexadecene) were also detected in other extracts. As cited earlier Indole, bisindole, imidazole containing alkaloids and other several analogues have been reported from diverse sources exhibiting pharmacological activities such as antimicrobial, cytotoxic, antitumor and antiviral. In our study three compounds having imidazole and indolinone as functional group were detected as minor fractions in *H. fontinalis* & *L. officinalis*. The imidazole or other derivative like imidazolidinedione detected in these extracts is capable of inducing such effects. In these extracts a pool of natural antioxidants like  $\alpha$ -tocopherol and an unusual neoacid (2,4-di-tert-butyl phenol/cyclohexanecarboxylic acid) which are reported from plants as well as in marine algae [15],[16] are said to demonstrate good antioxidant and fungicidal properties which occurs in natural peptides and natural alkaloids in cyanobacteria, fungi and in other microbes [17] were present in 50% of the extracts. In this study, six typical steroidal components present were 22, 23-Dibrostigmasterol, Stigmast-5-en-3-ol ( $\beta$ -Sitosterol), Stigmast-5,22-dien-3-ol ( $\beta$ -Stigmasterol), Cholest-5-en-3-ol, Ergost-5-en-3-ols (campesterol) and Stigmast-5-en-3-yl-9-octadecanoate. These sterols predominantly possess several pharmacological properties. Of these sterols  $\beta$ -Sitosterol is known to have antifungal activity and inhibit carcinogenesis [18, 19], ergosterol, sitosterol and stigmasterol reportedly has antifungal activity [20]. Several other compounds having furan and quinone structures, aldehydes like octadecenal and esters of citronellol and glycerol were present in minute amounts. Majority of cyanobacterial species also contains small amounts of short-chain hydrocarbons [21] like heptadecane. Small amounts of tetradecane (0.56 & 1.02%) were detected in *Chroococcus* Sp. and *Scytonema* Sp. while heptadecane was detected as 0.33 & 20.95% in *L. officinalis* & *Anabaena* Sp. respectively. Among the least detected compounds were octadecanethiol, 1-naphthalenepentanoic acid, scopolamine and three different nucleoside sugar moiety. The primary aim of this study was to reveal the bioactivity of these Cyanobacterial species. Diethyl ether extracts of these cyanobacterial species were tested against 24 human bacterial pathogens by agar well diffusion assay. It revealed, pronounced antibacterial activity against Gram positive bacteria by all extracts except in *Chroococcus* and *Anabaena* Sp. Anti bacterial activity against Gram positive bacteria was more frequent than against Gram negative bacteria. These extracts possessed different intensity of antibacterial action seen as in inhibition zones. This variation in inhibition zones might be attributed due to the difference in total pool of active metabolites present in these extracts. High contents of active fatty acids in these extracts of cyanobacteria are responsible for antimicrobial effects. Total content of saturated and unsaturated fatty acids ranged between 2.4 to 23.36% and 14.22 to 46.47% respectively. Fatty acids largely known in cyanobacteria are Octadecanoic, Octadecenoic, hexadecanoic acids, hexadecenoic acids, hexadecadienoic [21]. The fatty acids 9,12-octadecadienoic (Linoleic); 9,12,15-octadecatrienoic ( $\alpha$ -Linolenic) along with saturated fatty acids (tetradecanoic, heptadecanoic, eicosanoic and docosanoic acid) were present in these extracts affording antimicrobial and cytotoxic action. Recently, linoleic,  $\alpha$ -Linolenic acids have been reported to have growth inhibitory and proapoptotic effects [22], [23]. In addition to these fatty acids three isomers of 1, 2-benzenedicarboxylic acid (dioctyl, diisooctyl & ethylhexyl esters) were detected. Though in nature dicarboxylic acids are found as components of polymeric compounds in plants but some unusual dicarboxylic acids (short-chain, hydroxyl, branched, saturated and dioic acids) have also been reported from the cyanobacteria *Aphanizomenon* [24] as minor contents. They have also been reported in water hyacinth (1,2 benzenedicarboxylic acid esters  $M_w$  -390) [25]. Dicarboxylic acids such as 1, 2-Benzenedicarboxylic acid are also known to have antibacterial and anti-proliferative potential. Here, in our study at least one among the three isomers of this compound was present in

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all extracts except in *A.ambigua*. The antibacterial action by *A.ambigua* extract remained qualitatively comparable to *A. Fertillisma*, *A. Variabilis*, *W. prolifica* and *Scytonema* Sp. having low to high content (0.56-11.22%) of these compounds. However, the absence of such compounds in *A.ambigua* showed lowest toxicity ( $LD_{50}$ 146 $\mu$ g/ml) against HeLa cell line indicating the importance of this compound as cytotoxic. The high relative content of 1,2-Benzenedicarboxylic acid dioctyl in *A. fertillisma* (10.64%) & and ethyl hexyl ester in *Scytonema* Sp.(11.22%) is evidenced with higher cytotoxicities even at lower concentration of extracts against both cell line HeLa and SiHa. It was also noted that *A.ambigua* had comparatively high content of the sesquiterpenoid compounds also known as 2,6,11 & 2,6,10 trimethyl dodecanes (farnesol). This compounds have proven antibacterial action against *S.aureus*, MRSA (Methicillin Resistant *S. aureus*) & MSSA (Methicillin Susceptible *S. aureus*) [26] and fungal growth inhibition [27]. Farnesol has the ability to sensitize *S. aureus* to antimicrobials when used in synergy with antimicrobials. Therefore, it has been suggested for a potential application as an adjuvant therapeutic agent against biofilm related infections involving MRSA and thereby counteracting drug resistance. Farnesol is also known to possess chemopreventive and anticancerous activity by unknown mechanisms. The compounds belonging to imidazole and indolinones have been previously known for better pharmacological properties such as antibacterial, antifungal and anti-carcinogenesis. Further, the presence of the antioxidants, (alpha- tocopherol), tert-butyl containing compound, gamma butyrolactone and phytosterols are capable of inducing such effects. The heavy presence of sitosterol and stigmasterol seems to potentiate such effects. The aldehyde octadecenal and volatile alkanes (heptadecane and tetradecane) are also capable of such activity. The aldehydes (Butanal, heptanal and octanal) have been reported to show biomedical effects [28], [29], [30]. A large fraction of heptadecane was reported in ethyl acetate extracts of three blue green algae (*A.variabilis*, *A.flosaquae* and *O.angustissima*) exhibiting antibacterial activity [31].

## IV. CONCLUSION

Several natural products can be explored for the huge needs of novel compounds having medicinal applications. Based on these results it can be concluded that these fresh water strains of cyanobacteria are abundant sources of several pharmaceutically important compounds which can be used as sources of new and useful antimicrobial chemical entities against bacteria. The chemicals present in these extracts are cytotoxic against cervix cancer cell lines which can be potentially explored further.

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